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(54) Title: RIBOZYME THERAPY FOR HEPATITIS B INFECTION

(57) Abstract

This invention provides ribozymes useful to treat or prevent Hepatitis B virus ("HBV") infection in an organism or subject, as well as methods of treating an HBV infection. Reagents such as vectors, host cells, DNA molecules coding for these ribozymes useful in methods of treatment and prevention of HBV infection also are provided.

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RIBOZYME THERAPY FOR HEPATITIS B INFECTION

BACKGROUND OF THE INVENTION

Hepatitis B viral (HBV) infection remains a worldwide health problem, with estimates of over 300 million chronically infected individuals. The Center for Disease Control (CDC) estimates that there are approximately 1 million carriers in the United States and over 300,000 new infections per year. Chronic HBV infection is a major cause of liver cirrhosis. HBV infection also is associated with one of the most common visceral malignancies worldwide, primary hepatocellular carcinoma. In areas of the world where HBV infection is endemic, hepatocellular carcinoma (HCC) is among the most common lethal malignancies.

Hepatocellular carcinoma is a rapidly fatal tumor unless curative hepatic resection is possible. Unfortunately, most patients with HBV and HCC are cirrhotic and resection is contraindicated. Chemotherapy also has proven to be ineffective. Chemoembolization has shown some recent promise in uncontrolled trials. Orthotopic liver transplantation (OLT) has been performed for HCC, but the long term survival is less than 25%. Many centers have abandoned orthotopic liver transplantation to treat hepatocellular carcinoma because of the poor outcome and the obligate loss of the donated organ at this time, when there are two suitable recipients for every donor organ.

Alfa 2b interferon has been successfully used to treat chronic HBV infection in adults and is now being studied in children. Prognostic indicators of success include a low rate of viral replication and a substantial elevation of serum aminotransferase enzymes. In chronic carriers and patients with modest aminotransferase enzyme elevations, however, treatment is usually unsuccessful. Interferon is also ineffective in immunosuppressed patients with recurrent HBV infection post-OLT. Thus, a need exists

for an effective treatment to combat HBV infection. This invention satisfies this need and provides related advantages as well.

SUMMARY OF THE INVENTION

This invention provides ribozymes useful to treat or prevent Hepatitis B Virus ("HBV") infection in an organism or subject, as well as methods of treating an HBV infection. Reagents such as vectors, host cells, DNA molecules coding for these ribozymes useful in methods of treatment and prevention of HBV infection also are provided.

DETAILED DESCRIPTION OF THE INVENTION

The viral genome of HBV is about 3000 to 3300 nucleotides in length. Although the viral DNA is circular, both strands of the viral duplex are linear, and the circular conformation is maintained by extensive base-pairing between the two gapped DNA strands. The negative strand of the virus is about 3200 nucleotides in length while the positive strand is shorter, due to single-stranded gaps of variable size. Upon infection, the HBV DNA polymerase in the nucleocapsid core is activated and completes the synthesis of the positive strand, using the negative strand as a template. These features of the genome structure have suggested a retrovirus-like pathway of virus replication.

After conversion of the gapped double stranded DNA to fully double stranded DNA, a full-length positive-strand RNA is transcribed from the DNA template. It is believed that this pregenome RNA is packaged within the cell to form an "immature core," and by reverse transcription, a DNA strand of negative polarity is synthesized. This step is followed by the synthesis of a

partial positive strand and the full maturation of the virus particle containing a gapped DNA genome. However, it is the pregenome RNA and the mRNA of the virus which is the target of the ribozymes of this invention. Successful inhibition at this stage in the viral cycle can potentially eliminate hepatic and extrahepatic replication and transmission in all infected patients, including chronic carriers. An additional benefit is the prevention of Hepatitis D viral infection which requires HBsAg as its envelope.

As used herein, "ribozymes" are intended to include RNA molecules that contain anti-sense sequences for specific recognition, and a RNA-cleaving enzymatic activity. The catalytic strand cleaves a specific site in a target RNA at greater than stoichiometric concentration. Two "types" of ribozymes are particularly useful in this invention, the hammerhead ribozyme (Rossi, J.J. et al., Pharmac. Ther. 50:245-254 (1991) incorporated herein by reference) and the hairpin ribozyme. (Hampel et al., Nucl. Acids Res. 18:299-304 (1990) and U.S. Patent No. 5,254,678, issued October 19, 1993, each incorporated herein by reference.) Because both hammerhead and hairpin ribozymes are catalytic molecules having antisense and endoribonucleotidase activity; ribozyme technology has emerged as a potentially powerful extension of the antisense approach to gene inactivation.

Accordingly, this invention provides a ribozyme having the ability to inhibit replication and infectivity of a hepatitis B viral infection in a cell. This ribozyme is a hammerhead (for example, as described by Forster and Symons (1987) Cell 48:211-220; Haseloff and Gerlach (1988) Nature 328:596-600; Walbot and Bruening (1988) Nature 334:196; Haseloff and Gerlach (1988) Nature 334:585, each incorporated herein by reference) or a hairpin ribozyme (for example, as described by Haseloff et al., U.S. Patent

No. 5,254,678, issued October 19, 1993 and Hempel et al., European Patent Publication No. 0 360 257, published March 26, 1990, each incorporated herein by reference) having the ability to specifically target, cleave and inactivate HBV pregenome RNA or mRNA. For example, the ribozyme specifically targets, cleaves and inactivates the HBV X protein, which is the most potent regulatory protein for HBV. In a separate embodiment, the ribozyme target is the RNA coding for the surface antigen of HBV.

The sequence requirement for the hairpin ribozyme is any RNA sequence consisting of NNNG/CN*GUCNNNNNNNN (where N*G is the cleavage site, and where N is any of G, U, C, or A). The sequence requirement at the cleavage site for the hammerhead ribozyme is any RNA sequence consisting of NUX (where N is any of G, U, C, or A and X represents C, U or A) can be targeted. Accordingly, the same target within the hairpin leader sequence, GUC, is useful for the hammerhead ribozyme. The additional nucleotides of the hammerhead ribozyme or hairpin ribozyme is determined by the target flanking nucleotides and the hammerhead consensus sequence. See Ruffner et al. (1990) Biochemistry 29:10695-10702, incorporated herein by reference. This information, and the published sequence of the HBV genome and RNA encoding surface antigen of HBV enables the production of the ribozymes of this invention. Appropriate base changes in the ribozyme is made to maintain the necessary base pairing with the target HBV sequences. The sequence of the HBV is well known to those of skill in the art and can be obtained from GenBank, the contents of which are incorporated herein by reference.

Cech et al. (U.S. Patent No. 4,987,071, issued January 22, 1991) has disclosed the preparation and use of certain synthetic ribozymes which have endoribonuclease activity. These ribozymes are based on the properties of the Tetrahymena ribosomal RNA self-splicing reaction and

require an eight base pair target site with a requirement for free guanosine or guanosine derivatives. A temperature optimum of 50°C is reported for the endoribonuclease activity. The fragments that arise from cleavage contain 5′ phosphate and 3′ hydroxyl groups and a free guanosine nucleotide added to the 5′ end of the cleaved RNA. In contrast, the ribozymes of this invention hybridize efficiently to target sequences at physiological temperatures, making them suitable for use in vivo, not merely as research tools (see, column 15, lines 18 to 42, of Cech et al., U.S. Patent No. 4,987,071).

The ribozymes of this invention and DNA encoding the ribozymes, described in more detail below, can be chemically synthesized using methods well known in the art for the synthesis of RNA molecules. (For example, according to recommended protocols of Promega, Madison, Wis., USA). The ribozymes also can be prepared from a DNA molecule (that upon transcription, yields an RNA molecule) operably linked to an RNA polymerase promoter, e.g., the promoter for T7 RNA polymerase or SP6 RNA polymerase. Accordingly, also provided by this invention is a nucleic acid molecule, i.e., DNA or cDNA, coding for the ribozymes of this invention. When the vector also contains an RNA polymerase promoter operably linked to the DNA molecule, the ribozyme can be produced in vitro upon incubation with RNA polymerase and nucleotides. In a separate embodiment, the DNA is inserted into an expression cassette as described in Cotten and Birnstiel (1989) EMBO J. 8(12):3861-3866 and in Hempel et al., Biochemistry 28:4929-4933 (1989), each incorporated herein by reference. A more detailed discussion of molecular biology methodology is disclosed in Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, incorporated herein by reference. After synthesis, the RNA molecule can be modified by ligation to a DNA molecule having the ability to stabilize the ribozyme and make it resistant to

RNase. Alternatively, the ribozyme can be modified to the phosphothio analog for use in liposome delivery systems. This modification also renders the ribozyme resistant to endonuclease activity.

The DNA molecule also can be in a host procaryotic or eucaryotic cell in culture or in the cells of an organism. Appropriate procaryotic and eucaryotic cells can be transfected with an appropriate transfer vector containing the DNA molecule encoding a ribozyme of this invention. When the DNA molecule is operatively linked to a promoter for RNA transcription, the RNA can be produced in the host cell when the host cell is grown under suitable conditions favoring transcription of the DNA molecule. The vector can be, but is not limited to a plasmid, a virus, a retrotransposon or a cosmid. Because HBV is an acute or chronic infection of the liver, hepatotrophic vectors are preferred. Examples of such vectors are disclosed in U.S. Patent No. 5,166,320, incorporated herein by reference. To treat chronic HBV infection, the hepatitis delta vector or live, attenuated delta virus can be used. To treat acute HBV infection, an adenoviral vector or an adeno-associated vector type 1 ("AAV-1") or adeno-associated vector type 2 ("AAV-2") are particularly useful. Methods of gene therapy are well known in the art, see, for example, Larrick, J.W. and Burck, K.L. Gene Therapy: Application of Molecular Biology, Elsevier Science Publishing Co., Inc. New York, New York (1991) and Kreigler, M. Gene Transfer and Expression: A Laboratory Manual, W.H. Freeman and Company, New York (1990), each incorporated herein by reference.

To produce the ribozymes with a vector, the nucleotide sequences coding for ribozymes are placed under the control of a strong promoter such as the lac, SV40 late, SV40 early, or lambda promoters. Ribozymes are then produced directly from the transfer vector in vivo.